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The role of transport processes in survival of lactic acid bacteria

Energy transduction and multidrug resistance

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Abstract

Lactic acid bacteria play an essential role in many food fermentation processes. They are anaerobic organisms which obtain their metabolic energy by substrate phosphorylation. In addition three secondary energy transducing processes can contribute to the generation of a proton motive force: proton/substrate symport as in lactic acid excretion, electrogenic precursor/product exchange as in malolactic and citrolactic fermentation and histidine/histamine exchange, and electrogenic uniport as in malate and citrate uptake in *Leuconostoc oenos*. In several of these processes additional H^+ consumption occurs during metabolism leading to the generation of a pH gradient, internally alkaline. Lactic acid bacteria have also developed multidrug resistance systems. In *Lactococcus lactis* three toxin excretion systems have been characterized: cationic toxins can be excreted by a toxin/proton antiport system and by an ABC-transporter. This cationic ABC-transporter has surprisingly high structural and functional analogy with the human MDR1-(P-glycoprotein). For anions an ATP-driven ABC-like excretion systems exist.

Abbreviations: pmf – proton motive force, smf: sodium ion motive force.

Introduction

Lactic acid bacteria are the most important bacteria used in food fermentations. Examples are fermented milk products, including cheese, fermented vegetables such as sauerkraut and soy sauce and fermented sausages. They also play crucial roles in other fermentation processes such as the production of wine. Many of these fermentation processes date back to prehistoric times. One of the first microbiologists who realised the importance of lactic acid bacteria for the food industry and who studied these bacteria extensively was M.W. Beyerinck. In the beginning of this century he published two classical papers on the industrial lactic acid fermentation (Beyerinck 1901) and on the process of lactic acid fermentation in milk (Beyerinck 1907). In the last 15 years the research on lactic acid bacteria has mainly intensified as a result of an European community funded programme on lactic acid bacteria. This research activity has supplied a wealth of information about the systematics, genetics, physiology and fermentation technology of lactic acid bacteria and has led to a phase in which extensive manipulation of the important traits is possible in a very controlled way.

The studies on the physiology of lactic acid bacteria have focussed mainly on the metabolic pathways such as lactose fermentation, citrate metabolism and protein degradation. These studies have led to the realisation that translocation processes across the cytoplasmic membrane of the bacteria are crucial and often the rate controlling steps in metabolism. An example of such a crucial transport step in metabolism is the role of the oligopeptide transport system in the proteolysis of casein during cheese production (Kunji et al. 1995). Transport processes of amino acids, peptides and lactose have been studied in detail and this information

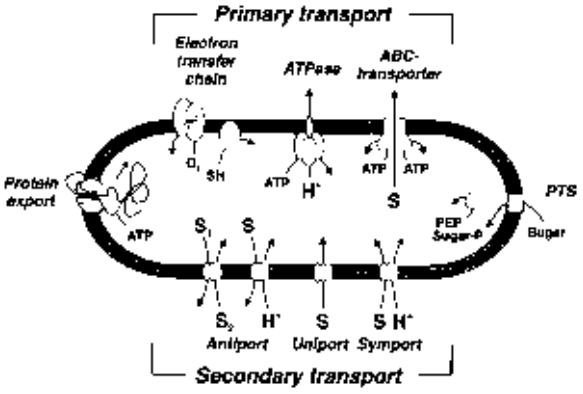

Figure 1. Schematic presentation of the transport processes in the cytoplasmic membranes of bacteria.

has been extensively reviewed (Konings et al. 1989, 1994). One rather unexpected outcome of these studies is that transport processes turn out to play a crucial role in survival under energy-limited conditions or in environments in which toxic compounds are present. This review will discuss a number of transport phenomena which are important for survival of lactic acid bacteria e.g. energy transduction by secondary transport processes and multidrug resistance processes.

Energy transduction in bacteria

For all living cells it is essential to transduce energy to a form that can be used to drive energy-requiring processes such as the synthesis of macromolecules, motility, uptake of solutes across the cytoplasmic membrane, etc. This energy is termed metabolic energy. There are in essence two forms of metabolic energy: energy-rich phosphate bond intermediates with ATP as the major representative and electrochemical energy stored in ion gradients, mainly proton or sodium-ion gradients. These two forms of metabolic energy can be obtained by substrate level phosphorylation and by chemiosmotic free energy-conserving processes (Mitchell 1968). The free energy released in a particular enzymatic reaction is directly used in the process of substrate level phosphorylation for the synthesis of ATP, which functions as a universal molecular carrier of metabolic energy. In chemiosmotic free energy conservation chemical, light or redox energy is converted into electrochemical energy by coupling the chemical reactions to vectorial translocation of ions across the membrane. In most bacteria these ions are protons, but in a number of bacteria sodium-ions are the major coupling ions in energy transducing processes (Lolkema et al. 1994). The systems which generate these electrochemical ion gradients are the 'primary transport systems' and the process is 'primary metabolic energy generation' (Figure 1). The electron transfer systems (respiratory chains, anaerobic electron transfer systems, phototrophic light-driven electron transfer systems) and the membrane-bound F_0F_1-ATPases are the best studied representatives of primary transport systems. The electrochemical gradient of a particular ion exerts a force on these ions which is termed the ion motive force, i.e. proton motive force (pmf) or sodium ion motive force (smf). The membrane bound electron transfer systems are mainly responsible for pmf or smf generation in bacteria that can use light or redox energy. In lactic acid bacteria the proton motive force contributes to the forces involved in the transport step of most secondary transport processes. The pmf is composed of two components: an electrical potential ($\Delta\Psi$), and a chemical potential of protons (- ZΔpH; Z = 2.3RT/F) across the membrane according to the following equation:

$$pmf = \Delta\Psi - \frac{2.3RT}{F}\Delta pH \quad (mv)$$

Secondary transport systems convert the (electro)-chemical energy of one solute into the (electro)-chemical energy of another solute. They are classified in three groups (Figure 1) (i) *uniporters* catalyse the translocation of only one solute across the cytoplasmic membrane; (ii) *symporters* mediate the coupled transport of two or more solutes in the same direction; (iii) *antiporters* couple the movement of one solute in one direction to the translocation of another solute in the opposite direction. Usually, but not necessarily, one of the solutes in the coupled translocation process is a proton. The solutes which are transported by the secondary transport systems can be neutral or negatively or positively charged. Furthermore, the number of protons translocated per solute can vary. Consequently, the forces which drive the translocation processes differ (see Figure 2). It should be emphasised that the direction of net translocation is determined by the direction of the driving force unless other regulatory factors prevent such an action.

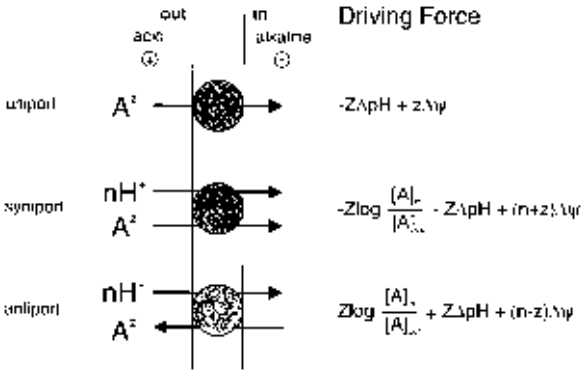

Figure 2. Driving forces for three secondary transport processes. A^Z is the transported solute with charge z. Z log $[A]_{in}/[A]_{out}$ represents the contribution of the chemical gradient of A across the membrane in mV to the driving force of A. n represents the number of translocated protons.

The generation of metabolic energy by secondary transport processes

In proton-dependent symport and antiport systems the pmf or one of its components is used to drive the accumulation or extrusion of another solute (Poolman & Konings 1993). In this way a chemical gradient of the solute is generated which, at thermodynamic equilibrium (total driving force = 0), will be equal and opposite to the driving force supplied by the proton motive force. Then, the chemical gradient of solute A (Z log $[A]_{in}/[A]_{out}$) is equal, but opposite in sign, to the driving force exerted by the components of the pmf. If for some reason the chemical gradient of A exceeds the driving force supplied by the proton motive force the reversed process can occur and the solute gradient will drive the process in the opposite direction. This reversed transport will lead to the generation of a pmf. Thus, in principle, the activity of a secondary transport system can lead to the generation of a proton motive force, which, subsequently, can drive other metabolic energy-requiring processes such as ATP synthesis by the membrane bound F_0F_1-ATPase. Studies in lactic acid bacteria have revealed that these latter processes play an important role in the bioenergetics of these organisms (Otto et al. 1980; Poolman 1990; Konings et al. 1994, 1995; Lolkema et al. 1996). Substrate uptake as well as product excretion can contribute to energy conservation in the form of the proton motive force. If the overall process involves the net translocation of charge, the electrical potential across the membrane will be affected; when protons are translocated the

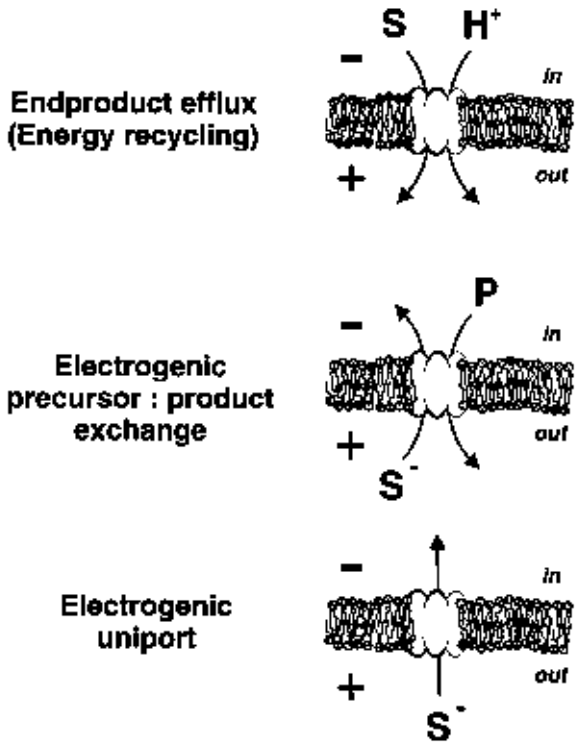

Figure 3. Proton motive force generating secondary transport processes.

pH-gradient across the membrane will be changed. In several of the processes which will be discussed below the transport process is followed by metabolism in the cytoplasm of the accumulated solute (precursor). If in this metabolism net protons are consumed, an additional generation of a ΔpH will occur.

Excretion of lactic acid

One mechanism by which a pmf can be generated is by electrogenic excretion of an end product in symport with proton(s) (Figure 3). An example is found in the excretion of lactic acid in lactic acid bacteria. During glucose or lactose fermentation lactic acid is produced that can be excreted via a secondary transport system. The excretion process in *Lactococcus lactis* has been studied *in vivo* (Otto et al. 1980; Ten Brink & Konings 1982; Ten Brink et al. 1985) and in membrane vesicles (Otto et al. 1982) within the pH range 5 to 8. The apparent pK of lactic acid is 3.9, and at high intracellular pH values essentially all lactic acid is in the anionic form. At high pH values the excretion process was found to result in the generation of a membrane potential, inside

negative. This shows that the process is electrogenic, indicating that more than one proton is translocated per lactate anion. At low pH values lactate excretion does not generate a membrane potential. The excretion is an electroneutral process and only one proton is translocated per lactate anion. These observations indicate that at high pH values lactate is excreted by a proton symport system with two or more protons (Konings & Booth 1981) while at low pH values excretion occurs via the same transport system but with a proton/lactate stoichiometry of one and/or by passive diffusion of the undissociated lactic acid. The degradation of glucose by the glycolytic pathway leads to the formation of lactic acid (lactate anion plus one H^+). Therefore, only excretion of lactate in symport with two or more protons will lead to the generation of a pmf and contribute to additional metabolic energy during the fermentation process. The process in which excretion of metabolic end-products via ion symport mechanisms results in the generation of metabolic energy has been termed 'energy recycling' (Michels et al. 1979).

Decarboxylation processes

Several exchange systems in bacteria mediate the net translocation of charge across the cytoplasmic membrane which results in the generation of a membrane potential. If, in addition, in the conversion of substrate to product a proton is consumed the fermentation will lead to an increase of the internal pH and, consequently, to the formation of a ΔpH (Lolkema et al. 1995). Then, the pathway generates a proton motive force consisting of both a membrane potential and pH gradient (Figure 3). Most known examples of such pathways to date are precursor/product exchange systems in combination with precursor decarboxylation. The best studied examples of decarboxylation driven pathways in lactic acid bacteria are malate/lactic acid exchange (malolactic fermentation) in *L. lactis* (Poolman et al. 1991), histidine/histamine exchange in *Lactobacillus buchneri* (Molenaar et al. 1993) and citrate/lactate exchange (citrolactic fermentation) in *Leuconostoc mesenteroides* (Marty et al. 1995, 1996). These pathways are driven by the decarboxylation reaction ($\Delta G_0' \approx$ - 25 kJ/mol). The translocation process is driven by the inwardly directed substrate concentration gradient and the outwardly directed product concentration gradient. Both gradients are maintained by the decarboxylation reaction.

Malolactic fermentation is carried out by some species of the genera *Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus* (Kunkee 1991). In this pathway L-malate enters the cells and is subsequently decarboxylated by malolactic enzyme to yield L-lactic acid and carbon dioxide, after which the reaction products leave the cell (Figure 4A). As a consequence of the pK differences of the carboxylic acid groups on the substrate and products, the pathway results in alkalinization of the medium. It has been shown for *L. lactis* that L-malate utilization results in the formation of a pmf that is sufficiently high to drive ATP synthesis via the F_0F_1-ATPase (Poolman et al. 1991).

The decarboxylase catalyses the conversion of L-malate into L-lactate in two steps. First, L-malate is oxidatively decarboxylated to yield pyruvate while the electrons are donated to NAD^+ which is a cofactor of the enzyme. In the second step, pyruvate which remains bound to the enzyme, is reduced by NADH to L-lactate. Recently, the structural gene coding for the enzyme of *Lactococcus lactis* has been cloned and sequence comparison shows that malolactic enzyme is homologous to the malic enzymes that catalyse the conversion of malate into pyruvate (Anasany et al. 1993). The transport of L-malate has been characterized in artificial membranes prepared from *L. lactis* IL1403 (mal^+) and in isogenic mutants, that are defective in either L-malate transport or L-malate decarboxylation (Poolman et al. 1991). Membrane vesicles catalyse unidirectional uptake of L-malate (either as $Hmal^-$ uniport or mal^{2-}/H^+ symport) and L-malate/L-lactate exchange (either $Hmal^-$/Hlac or mal^{2-}/lac^-). In both cases a negative charge is translocated into the membrane vesicle which results in the formation of a membrane potential (inside negative). L-malate accumulates inside the membrane vesicles in the presence of a pH gradient because of deprotonation of the transported species in the more alkaline internal milieu. The exchange of L-$malate^{2-}$ for L-$lactic^-$ acid is much more rapid than L-$malate^{2-}/H^+$ symport, and the former reaction is likely to be most relevant under physiological conditions of malolactic fermentation in *L. lactis*. Since charge compensation in the decarboxylation reaction requires the consumption of a proton, the cytoplasm is alkalinized relative to the outside medium and a ΔpH is formed. Thus, the free energy of the decarboxylation reaction is converted into a pmf simply by compartmentalization of the malolactic fermentation pathway, i.e. stoichiometric uptake of L-$malate^{2-}$ and excretion of L-$lactic^-$ into the medium combined with decarboxylation in the cytoplasm.

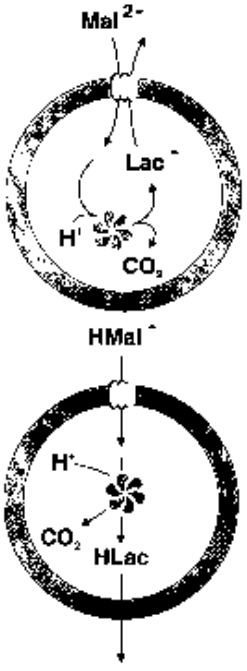

Figure 4. Proton motive force generation by malolactic fermentation in A. *Lactococcus lactis* and B. *Leuconostoc oenos*.

Histidine decarboxylation. *Lactobacillus buchneri* decarboxylates histidine to the biogenic amine histamine, which is excreted into the medium (Molenaar et al. 1993). The pK_A's of the imidazole ring of histidine and histamine are 6.0 and 5.8, respectively, and the protonation state of the side chain of both molecules will have a similar dependency on pH. However, histamine carries one additional positive charge which makes the histidine/histamine exchange electrogenic, resulting in the net movement of a positive charge to the outside. A ΔpH is formed in a similar manner as observed for malolactic fermentation.

Citrolactic fermentation. Cometabolism of glucose and citrate by the lactic acid bacterium *Leuconostoc mesenteroides* results in a growth advantage relative to growth on glucose alone. Citrate is taken up by a secondary transporter, the product of the *citP* gene and, subsequently, converted to oxaloacetate and acetate by citrate lyase. Oxaloacetate is decarboxylated by the cytoplasmic enzyme oxaloacetate decarboxylase yielding CO_2 and pyruvate. Pyruvate from citrate promotes a metabolic shift in the heterofermentative pathway for glucose breakdown yielding additional ATP which usually is put forward to explain the increased growth properties (Cogan 1987; Hugenholtz 1993; Lin et al. 1991; Schmitt et al. 1990). In the absence of citrate, acetyl-P formed from glucose is reduced to ethanol which balances the redox equivalents produced in the other steps of the phosphoketolase pathway. In the presence of citrate, the redox equivalents are shuttled to pyruvate produced from citrate, yielding D-lactate and acetyl-P is converted into acetate via the acetate kinase pathway which results in the production of ATP. Recent studies have demonstrated that citrate breakdown in *Lc. mesenteroides* is a secondary metabolic energy generating pathway which provides an additional explanation for the growth enhancement (Marty-Teysset et al. 1995, 1996; Lolkema et al. 1996). Citrate enters the cell in exchange with D-lactate that is produced inside the cell as an end product during glucose/citrate cometabolism (citrolactic fermentation). The exchange is electrogenic and translocates negative charge into the cell thereby generating a membrane potential. The pathway as a whole is alkalinizing and results in a pH gradient, inside alkaline, over the cell membrane. Therefore, citrate metabolism in *Lc. mesenteroides* results in the generation of a proton motive force that consists of both a membrane potential and pH gradient.

Uniport systems. Studies of malate and citrate metabolism in *Leuconostoc oenos* have revealed that a proton motive force can also be generated by electrogenic uniport of a negatively charged substrate (Figure 3). *L. oenos* grows at more acidic pH values than for instance *Lactococcus lactis*. Malolactic fermentation, in this bacterium proceeds through the uptake of negatively charged mono-protonated $Hmalate^-$ by a uniport mechanism rather than by $Hmalate^-$/Hlac (or mal^{2-}/lac^-) exchange (Figure 4B). Lactic acid formed in the proton consuming decarboxylation reaction leaves the cell by passive diffusion. Energetically, the result is the same, i.e. generation of a membrane potential and pH gradient. Again, the whole pathway is driven by the decarboxylase. The difference is in the transporter that in case of the uniport is only dirven by the substrate concentration gradient. Therefore, it is essential that a high solute concentration gradient is maintained by keeping the internal concentration of the solute low by rapid internal metabolism (Salema et al. 1994, 1996). The different physiological functions of the malate carriers of *L. lactis* and *Lc. oenos* are reflected in the kinetic properties of the transporters. In *L. lactis* malate/lactate exchange is catalysed much more efficiently than unidirectional uptake. In *Lc. oenos* the opposite is true.

Surprisingly, studies of citrate transport in isolated membrane vesicles of *Lc. oenos* showed the same mechanism as observed for malate uptake (Ramos et al. 1994). The citrate carrier catalyses uniport of monovalent H_2cit^- implying that citrate metabolism by *Lc. oenos* is involved in the generation of a membrane potential. Citrate metabolism in citrate-fermenting lactic acid bacteria results in alkalinization of the medium, i.e. protons are consumed in the metabolic breakdown inside the cell and a pH gradient is formed. Therefore, citrate metabolism in *Lc. oenos* is predicted to generate a proton motive force consisting of a membrane potential generated during the electrogenic uptake of H_2cit^- and a pH gradient generated by the consumption of scalar protons in the further degradation of citrate inside the cell. Studies with resting cells of *Lc. oenos* confirmed the prediction made based on the studies with the membrane vesicles. Addition of citrate to the cells resulted in the generation of both a membrane potential and pH gradient independent of F_0F_1 H^+-ATPase activity.

Multidrug resistance

Toxic compounds have always been part of the natural environments of bacteria. The development of versatile mechanisms to resist antibiotics and other cytotoxic drugs has been crucial for the survival of the organisms (Lewis 1994; Nikaido 1994). An important group of resistance mechanisms is found in the specific drug resistance systems (SDR) and the multidrug resistance systems (MDR). These systems prevent the entrance of specific (SDR) or a variety (MDR) of toxic compounds into the cell by actively transporting the compounds out of the cell. MDR systems from eukaryotic organisms are studied extensively for reason that these systems cause resistance of carcinoma cells against chemotherapeutic agents (Simon & Schindler 1994). In bacteria both SDR and MDR systems are subject to extensive research since these systems are often responsible for the antibiotic resistance of pathogenic bacteria (Hayes & Wolf 1990).

In *L. lactis* three multidrug resistance systems have been discovered. One system confers resistance against anionic compounds; the other two systems are cation excretion systems (Van Veen et al. 1996a). On the basis of bioenergetic and structural criteria these transport systems can be divided into: (i) secondary drug transporters and (ii) ATP-binding cassette (ABC)-type drug transporters. The secondary transporters are single membrane proteins which mediate the extrusion of the drug in a coupled exchange with protons or sodium-ions (Poolman & Konings 1993). They are divided into two groups: (i) TEXANs (Toxin EXtruding ANtiporters) which have 12 to 14 transmembrane α-helical domains and (ii) Mini TEXANs with only 4 transmembrane α-helical domains (Schuldiner et al. 1995). The ABC-type drug transporters belong to the ABC superfamily, the members of which all contain a highly conserved ATP-binding cassette (Higgins 1992; Van Veen et al. 1996b). They utilize the energy released by ATP-hydrolysis to pump cytotoxic compounds out of the cell. Typically, ABC transporters require the function of four protein domains (Higgins 1992; Fath & Kolter 1993). Two of these domains are highly hydrophobic and consist each of six putative transmembrane α-helices. These hydrophobic domains form the path through which the substrate crosses the membrane. The other two domains are attached to the membrane at the cytoplasmic side. These domains bind ATP and couple ATP-hydrolysis to the translocation of the substrate (Dean et al. 1989; Amres et al. 1989).

Multidrug resistance in L. lactis

Extrusion of cations

L. lactis subsp. *lactis* MG1363 was found to be resistant to a number of toxic compounds such as ethidium bromide, daunomycin and rhodamine 6 G. To study this resistance phenomenon in detail mutant strains of *L. lactis* were isolated with resistance to high concentrations of these drugs. These mutants which were obtained by growing the organism on increasing concentrations of these toxic compounds, were termed EthR, DauR and RhoR, respectively (Bolhuis et al. 1994). These mutants were found to be cross resistant to a number of structurally and functionally unrelated drugs, among which are typical substrates for the mammalian multidrug transporter (MDR 1, P-glycoprotein) such as daunomycin, quinine, actinomycin D, gramicidin D and rhodamine 6G. Each of the multidrug resistant strains showed an increased resistance against the other two compounds for which resistant mutants were selected (Bolhuis et al. 1994). Furthermore, all three strains showed an increased rate of energy-dependent ethidium and daunomycin efflux compared to that of the wild-type strain and this efflux was drastically inhibited by the P-glycoprotein inhibitor reserpine. The energetics of the efflux process was analysed by study-

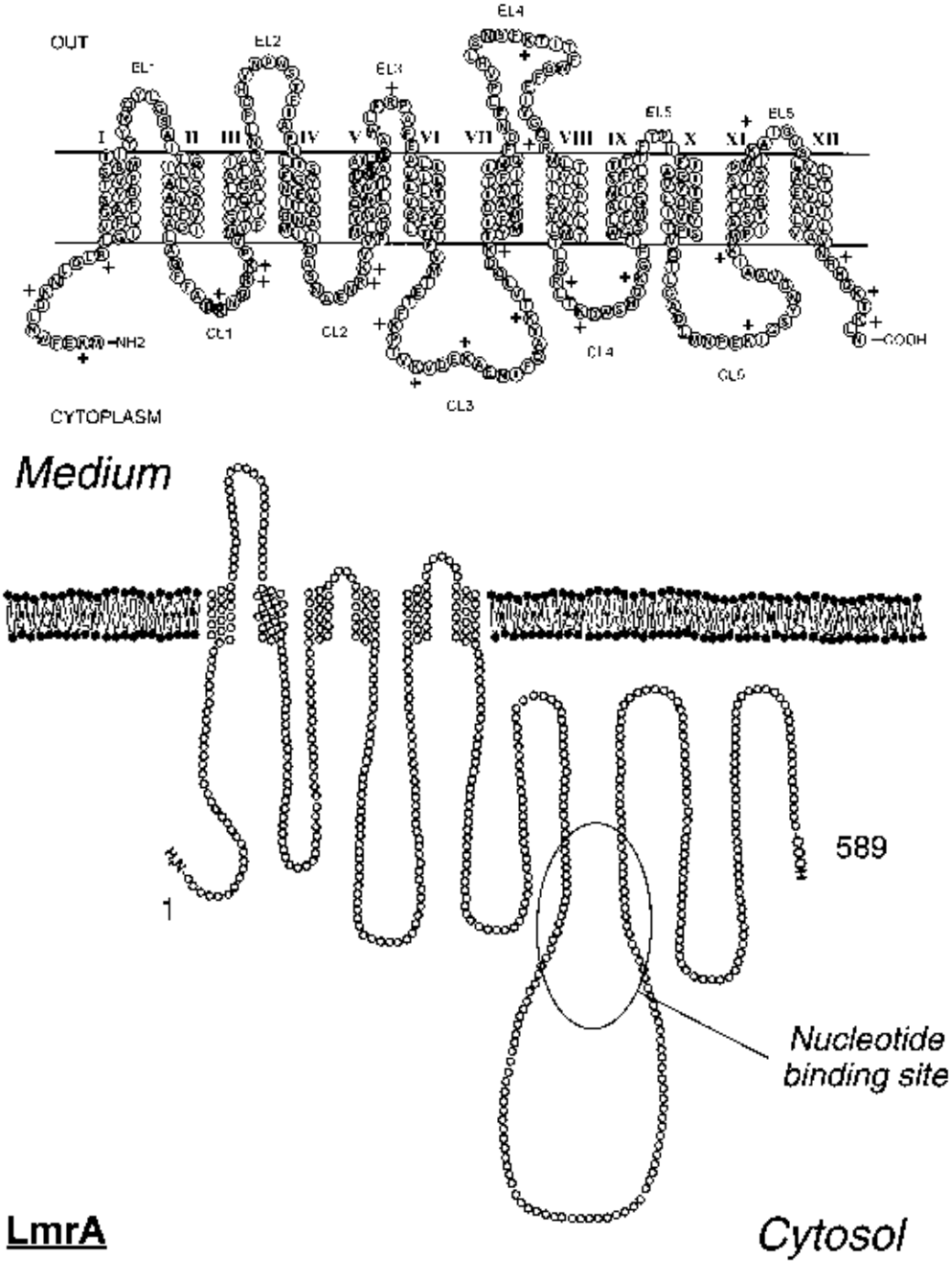

Figure 5. Secondary structure models of two drug extrusion systems in *Lactococcus lactis*. A. the toxin/proton antiport system LmrP and B. the ABC-drug extrusion system LmrA.

ing the effects on the efflux process of the pmf dissipating ionophores, valinomycin and nigericin, and the ABC transporter inhibitor ortho-vanadate. Ethidium efflux in the EthR strain was inhibited by ortho-vanadate and not by dissipation of the pmf, while both inhibitors decreased partially the efflux of ethidium in DauR and RhoR strains. From these results it was concluded that two cationic multidrug excretion systems are present in *L. lactis*: one which is pmf driven and a second one which is mainly present in the EthR strain and is ATP-dependent (Bolhuis et al. 1994). The energetic, genetic and biochemical properties of both systems have been studied in detail.

The pmf dependent drug efflux system is encoded by the *lmrP* gene, which encodes a hydrophobic polypeptide of 408 residues (Bolhuis et al. 1995). Hydropathy analysis suggest that this protein is composed of 12 transmembrane α-helices (Figure 5A). When expressed in *E. coli*, LmrP convers resistance to ethidium, daunomycin and tetraphenylphosphonium. The drug efflux process in *E. coli* or *L. lactis* in which LmrP was expressed, is driven by the pmf since ethidium efflux is completely inhibited by a dissipation of the pmf and is not affected by the ATPase inhibitor ortho-vanadate. From these studies and those in *E. coli* membrane vesicles containing LmrP protein (Bolhuis et al. 1996b), it is concluded that LmrP catalyses drug-proton antiport. It therefore belongs to the family of TEXANs.

When the *lmrP* gene was deleted, the mutant strain obtained still exhibited residual ethidium extrusion activity, which in contrast to the parent strain was inhibited by ortho-vanadate. This observation indicates that in the absence of the functional drug-proton antiport system LmrP, *L. lactis* is able to overexpress another, ATP-dependent drug extrusion system. This system was further characterised by cloning and functionally expressing the gene in *E. coli* (Van Veen et al. 1996b). The gene is designated *lmrA* and encodes for a protein of 589 amino acids with a molecular weight of 64 kDa. Hydropathy analysis of LmrA suggest the presence of an N-terminal hydrophobic domain with 6 putative transmembrane regions and a C-terminal hydrophilic domain (Figure 5B). The C-terminal domain contains the ATP-binding motif diagnostic of an ABC-type transporter. Most surprisingly, the LmrA protein was found to be very similar to the human P-glycoprotein MDR1. This latter protein is about double the size of LmrA (1280 amino acids) and is composed of two homologous halves each with 6 transmembrane regions and a large cytoplasmic loop containing the ATP-binding site. At the amino acid sequence level each half of P-glycoprotein has 48% similarity with LmrA.

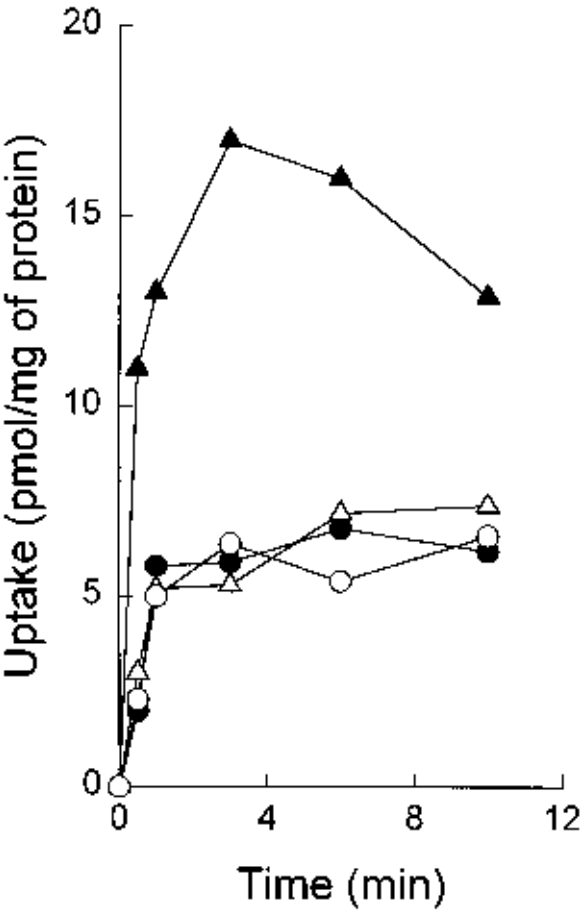

Figure 6. Uptake of daunomycin in inside-out membrane vesicles of *Escherichia coli* CS1562 (open symbols) and the strain in which LmrA was expressed (closed symbols). Uptake studies were done in the presence of valinomycin and nigericin (each at 1 nmol/mg of protein) and 1 mM ATP plus 0.1 mg/ml creatine kinase (▲, △) or 1 mM non-hydrolysable ATPγS (●, ○).

Expression of LmrA in *E. coli* resulted in an increased resistance of the organism against ethidium, daunomycin, rhodamine 6G and tetraphenyl phosphonium. The *E. coli* cells also excreted ethidium when glucose was added to cells which had previously accumulated ethidium in the absence of an energy source. Direct evidence that LmrA is an ATP-dependent drug extrusion system is obtained by studies in inside-out membrane vesicles from *E. coli* cells in which LmrA was expressed. In the presence of ATP but not in the presence of a pmf these membrane vesicles accumulat-

ed the substrates ethidium, daunomycin and ajmalinium, a high affinity substrate of P-glycoprotein (Figure 6). These results clearly identify LmrA as an ATP-dependent multidrug extrusion system. It represents the first example of a prokaryotic structural and functional homologue of P-glycoprotein.

Extrusion of anions

Besides the TEXAN LmrP and the P-glycoprotein homolog LmrA, *L. lactis* also possesses an ATP-dependent multidrug extrusion system with specificity for organic anions. This system was detected in cells of *L. lactis* due to its ability to extrude the fluorescent pH indicator BCECF (2′,7′-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein) (Molenaar et al. 1991, 1992). BCECF efflux is directed against a concentration gradient and strictly correlates with the cellular ATP concentration. In addition, BCECF efflux is strongly decreased in the presence of ortho-vanadate, a well known inhibitor of P-type ATPases and ABC transporters. Most convincingly, a UV mutant with a strongly reduced efflux rate could be isolated from a BCECF-loaded and lactose-energized cell population by selection of highly fluorescent cells in a flow cytometer-cell sorter (Molenaar et al. 1992). Studies in inside-out membrane vesicles of *L. lactis* have indicated that the BCECF transporter is not only able to transport carboxyfluorescein derivatives, but also glutathione conjugates such as dinitrophenyl-glutathione (Van Veen et al. 1996a). Interestingly, the substrate specificity of the BCECF transporter of *L. lactis* is remarkably similar to that of the human multidrug resistance-associated protein (MRP) (Müller et al. 1994; Leier et al. 1994; Zaman et al. 1994). The over expression of this ABC transporter in human cancer cells results in a P-glycoprotein-independent multidrug resistance phenotype. In hepatocytes, MRP plays a role in a glutathione S-transferase-dependent detoxification pathway for electrophilic drugs, and in the excretion of oxidized glutathione (GSSG) during oxygen stress (Ishikawa 1992). The BCECF transporter of *L. lactis* may have functions similar to those of MRP. Although *L. lactis* contains a manganese-dependent superoxide dismutase to detoxify oxygen radicals, the enzyme is not essential for the aerotolerance of this fermentative bacterium (Sanders et al. 1995). An alternative protection mechanism against oxygen damage may be based on the detoxification of oxygen radicals by reduced glutathione (Meister 1988). This compound is present at high concentrations in *L. lactis* and other facultative anaerobic bacteria (Fahey et al. 1978). During oxygen stress, optimal levels of reduced versus oxidized glutathione may be maintained through the excretion of GSSG via the BCECF transporter.

Molecular models of multidrug transport

Several models have been postulated for the pump function of multidrug transporters to explain their broad specificity for chemically unrelated compounds. Drug translocation may involve substrate transport from the cytoplasm to the exterior which would require an enormous flexibility of an ‘enzyme-like’ substrate recognition site (conventional transport hypothesis) (Altenberg et al. 1994; Mülder et al. 1993). Alternatively, multidrug transporters could recognize the lipophilic drugs by their physical property to intercalate into the lipid bilayer, and transport drugs from the lipid bilayer to the exterior (vacuum cleaner hypothesis) (Raviv et al. 1990), or from the inner leaflet to the outer leaflet of the lipid bilayer (flippase hypothesis) Higgins & Gottesman 1992; Higgins 1994).

Cells expressing LmrA were found to actively extrude the hydrophobic acetoxymethyl ester of BCECF (Bolhuis et al. 1996a). Similar observations on the human multidrug resistance P-glycoprotein were taken as evidence for the transport of hydrophobic compounds from the plasma membrane (Homolya et al. 1993). The transport mechanism of LmrA has been studied in greater detail by using the amphiphilic, fluorescent membrane probe TMA-DPH. Intercalation of TMA-DPH in the phospholipid bilayer is based on a fast partitioning of the probe in the outer leaflet, followed by a slower transbilayer movement of the probe from the outer to the inner leaflet of the membrane. The correlation between the initial TMA-DPH extrusion rate and the amount of TMA-DPH associated with the inner membrane leaflet, suggests that LmrA transports TMA-DPH from the inner leaflet of the cytoplasmic membrane (Bolhuis et al. 1996a). Thus, LmrA recognizes drugs by their hydrophobic properties as suggested by the ‘flippase’ and ‘vacuum cleaner’ models. Identical observations were made for LmrP mediated TMA-DPH transport in whole cells and in inside out membrane vesicles of *Escherichia coli*, suggesting a similar mechanism of drug extrusion by the secondary drug transporter of *L. lactis* (Bolhuis et al. 1996b).

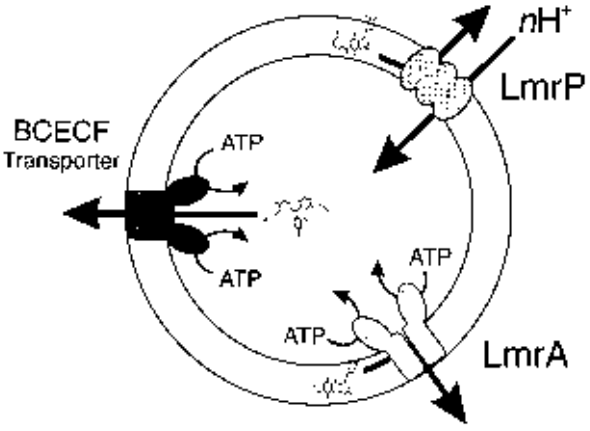

Figure 7. The three characterised multidrug resistance systems in *Lactococcus lactis*.

Conclusions

Lactic acid bacteria are considered to be rather simple organisms with limited physiological capacities. However, they have developed ingenious systems for extracting as much metabolic energy from their substrates as possible. Three classes of transport systems can play a role in this metabolic energy generation: symporters, antiporters and uniporters (Figure 3). The activity of these systems allow the lactic acid bacteria to survive under energy limited conditions.

Lactic acid bacteria have also developed extensive protection mechanisms against toxic compounds. In *Lactococcus lactis* three systems have been characterized: 2 against cationic compounds and one against anionic compounds (Figure 7).

These systems protect the organisms against toxic compounds in the environment, but they also might play a role in excreting toxic compounds produced during metabolism.

References

Altenberg GA, Vanoye CG, Horton JK & Reuss L (1994) Unidirectional fluxes of rhodamine 123 in multidrug resistant cells: Evidence against direct extrusion from the plasma membrane. Proc. Natl. Acad. Sci. USA 91: 4654–4657

Ames GF-L, Nikaido K, Groake J & Petithory J (1989) Reconstitution of periplasmic transport in inside-out membrane vesicles. Energization by ATP. J. Biol. Chem. 246: 3398–4002

Anasany V, Dequin S, Blondin B & Barre P (1993) Cloning, sequence and expression of the gene encoding the malolactic enzyme from *Lactococcus lactis*. FEBS Lett 332: 74–80

Beijerinck MW (1901) Sur les ferments lactiques de l'industrie. Archives Neerlandaises des Sciences Exactes et Naturelles, Serie II 6: 212–243

— (1907) On lactic acid fermentation in milk. Proceedings Royal Academy of Sciences, Amsterdam 10: 17–34

Bolhuis H, Molenaar D, Poelarends G, Van Veen HW, Poolman B, Driessen AJM & Konings WN (1994) Proton motive force-driven and ATP-dependent drug extrusion systems in multidrug-resistant *Lactococcus lactis*. J. Bacteriol. 176: 6957–6964

Bolhuis H, Poelarends G, Van Veen HW, Poolman B, Driessen AJM & Konings WN (1995) The lactococcal *lmrP* gene encodes a proton motive force-dependent drug transporter. J. Biol. Chem. 270: 26092–26098

Bolhuis H, Molenaar D, Van Veen HW, Poolman B, Driessen AJM & Konings WN (1996a) Multidrug resistance in *Lactococcus lactis*: evidence for ATP-dependent drug extrusion from the inner leaflet of the cytoplasmic membrane EMBO J. in press

Bolhuis H, Brands JR, Putman M, Van Veen HW & Konings WN (1996b) Energetics and mechanism of drug transport by the lactococcal mdr transporter LmrP (submitted)

Cogan TM (1987) Co-metabolism of citrate and glucose by *Leuconostoc* ssp.: effects on growth, substrates and products. J. Appl. Bacteriol. 63: 551–558

Dean DA, Davidson AL & Nikaido H (1989) Maltose transport in membrane vesicles of *Escherichia coli* is linked to ATP hydrolysis. Proc. Natl. Acad. Sci. USA 86: 4254–4260

Fahey RC, Brown WC, Adams WB & Worsham MB (1978) Occurrence of glutathione in bacteria. J. Bacteriol. 133: 1126–1129

Fath MJ & Kolter R (1993) ABC transporters: bacterial exporters. Microbiol. Rev. 57: 995–1017

Hayes JD & Wolf CR (1990) Molecular mechanisms of drug resistance. Biochem. J. 272: 281–295

Higgins CF (1992) ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. 8: 67–113

Higgins CF & Gottesman MM (1992) Is the multidrug transporter a flippase? Trends Biochem. Sci. 17: 18–21

Higgins CF (1994) Flip-Flop: The transmembrane translocation of lipids. Cell 79: 393–395

Homolya L, Holló Z, Germann UA, Pastan I, Gottesman MM & Sarkadi B (1993) Fluorescent cellular indicators are extruded by the multidrug resistance protein. J. Biol. Chem. 268: 21493–21496

Hugenholtz J (1993) Citrate metabolism in lactic acid bacteria. FEMS Microbiol. Rev. 12: 165–178

Ishikawa T (1992) The ATP-dependent glutathione S-conjugate export pump. Trends Biochem. Sci. 17: 463–468

Leier I, Jedlitschky G, Buchholz U, Cole SPC, Deeley RG & Keppler D (1994) The MRP gene encodes an ATP-dependent export pump for leukotriene C_4 and structurally related conjugates. J. Biol. Chem. 269: 27807–27810

Lin JP, Schmitt P & Divies C (1991) Characterization of a citrate-negative mutant of *Leuconostoc mesenteroides* subsp. *mesenteroides*: metabolic and plasmidic properties. Appl. Microbiol. Biotech. 34: 628–631

Konings WN, Poolman B & Driessen AJM (1989) Bioenergetics and solute transport in Lactococci. CRC Crit. Rev. Microbiol. 16: 419–476

Konings WN, Poolman B, Driessen AJM, Smid EJ & Molenaar D (1994) Mechanismes de transport des nutrients chez les bactéries lactiques. In: De Roissart H & Luquet F (Eds) Bactéria Lactiques, Volume 1, Chapters 1–5 (pp. 209–238) Editions Lavoisier, Paris

Konings WN & Booth IR (1981) Do the stoichiometries of ion-linked transport systems vary? Trends Biochem. Sci. 6: 257–262

Konings WN, Lolkema J & Poolman B (1995) The generation of metabolic energy by solute transport. Arch. Microbiol. 164: 235–242

Konings WN, Poolman B & Van Veen HW (1994) Solute transport and energy transduction in bacteria. Antonie van Leeuwenhoek 65: 369–380

Kunji ERS, Hagting A, De Vries CJ, Juillard V, Haandrikman AJ, Poolman B & Konings WN (1994) Transport of casein-derived peptides by the oligopeptide transport system is a crucial step in the proteolytic pathway of *Lactococcus lactis*. J. Biol. Chem. 270: 1569–1574

Kunkee R (1991) Some roles of malic acid in the malolactic fermentation in wine making. FEMS Microbiol. Rev. 88: 55–72

Lewis K (1994) Multidrug resistance pumps in bacteria: variations on a theme. Trends Biochem. Sci. 19: 119–123

Lin J, Schmitt P & Divies C (1991) Characterization of a citrate-negative mutant of *Leuconostoc mesenteroides* subsp. *mesenteroides*: metabolic and plasmidic properties. Appl. Microbiol. Biotech. 34: 628–631

Lolkema JS, Poolman BP & Konings WN (1995) The role of scalar protons in the generation of metabolic energy in lactic acid bacteria. J. Bioenerg. Biomem. 27: 467–473

— (1996) Secondary transporters and metabolic energy generation. In: Kaback HR, Konings WN & Lolkema JS (Eds) Transport Processes in Eukaryotic and prokaryotic membranes. Vol. II, Chapter XI (pp. 229–260) Elsevier Science, Amsterdam

Lolkema JS, Speelmans G & Konings WN (1994) Na^+-coupled versus H^+-coupled energy transduction in bacteria. Biochim. Biophys. Acta 1187: 211–215

Marty-Teysset C, Lolkema JS, Schmitt P, Divies C & Konings WN (1995) Membrane potential generating transport of citrate and malate catalyzed by the citP gene product of *Leuconostoc mesenteroides*. J. Biol. Chem. 270: 25370–25376

Marty-Teysset C, Posthuma C, Lolkema JS, Schmitt P, Divies C & Konings WN (1996) Proton motive force generation by citrolactic fermentation in *Leuconostoc mesenteroides*. J. Bact. 178: 2178–2185

Meister A (1988) Glutathione metabolism and its selective modification. J. Biol. Chem. 263: 17205–17208

Michels PAM, Michels JPJ, Boonstra J & Konings WN (1979) Generation of an electrochemical proton gradient in bacteria by the extrusion of metabolic end products. FEMS Microbiol. Lett. 5: 357–364

Mitchell P (1968) 'Chemiosmotic coupling and energy transduction'. Glynn Research Ltd., Bodmin, England

Molenaar D, Abee T & Konings WN (1991) Continuous measurement of the cytoplasmic pH in *Lactococcus lactis* with a fluorescent pH indicator. Biochim. Biophys. Acta 1115: 75–83

Molenaar D, Bolhuis H, Abee T, Poolman B & Konings WN (1992) The efflux of a fluorescent probe is catalyzed by an ATP-driven extrusion system in *Lactococcus lactis*. J. Bacteriol. 174: 3118-3124

Molenaar D, Bosscher JS, Ten Brink B, Driessen AJM & Konings WN (1993) Generation of a proton motive force by histidine decarboxylation and electrogenic histidine/histamine antiport in *Lactobacillus buchneri*. J. Bacteriol. 175: 2864–2870

Mülder HS, Van Grondelle R, Westerhoff HV & Lankelma J (1993) A plasma membrane 'vacuum cleaner' for daunorubicin in non-P-glycoprotein multidrug resistant SW-1573 human non-small cell lung carcinoma cells: a study using fluorescence resonance energy transfer. Eur. J. Biochem. 218: 871–882

Müller M, Meijer C, Zaman GJR, Borst P, Scheper RJ, Mulder HN, De Vires EG & Jansen PLM (1994) Overexpression of the gene encoding the multidrug resistance associated protein results in increased ATP-dependent glutathione S-conjugate transport. Proc. Natl. Acad. Sci. USA 91: 13033–13037

Nikaido H (1994) Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science 264: 382–388

Otto R, Lageveen RG, Veldkmap H & Konings WN (1982) Lactate efflux induced electrical potential in membrane vesicles of *Streptococcus cremoris*. J. Bacteriol. 146: 733–738

Otto R, Sonnenberg ASM, Veldkamp H & Konings WN (1980) Generation of an electrochemical proton gradient in *Streptococcus cremoris* by lactate efflux. Proc. Natl. Acad. Sci. USA 77: 5502–5506

Poolman B & Konings WN (1993) Secondary solute transport in bacteria. Biochim. Biophys. Acta 1183: 5–39

Poolman B, Molenaar D, Smid EJ, Ubbink T, Abee T, Renault PP & Konings WN (1991) Malolactic fermentation: electrogenic malate uptake and malate/lactate antiport generate metabolic energy. J. Bacteriol. 173: 6030–6037

Poolman B (1990) Precursor/product antiport in bacteria. Molec. Microbiol. 4: 1629–1636

Ramos A, Poolman B, Santos H, Lolkema JS & Konings WN (1994) Uniport of anionic citrate and proton consumption in citrate metabolism generate a proton motive force in *Leuconostoc oenos*. J. Bacteriol. 176: 4899–4905

Raviv Y, Pollard HB, Bruggemann EP, Pastan I & Gottesman MM (1990) Photosensitized labeling of a functional multidrug transporter in living drug resistant tumor cells. J. Biol. Chem. 265: 3975–3980

Salema M, Poolman B, Lolkema JS, Loureiro-Dias MC & Konings WN (1994) Uniport of monoanionic L-malate in membrane vesicles from *Leuconostoc oenos*. Eur. J. Biochem. 225: 289–295

Salema M, Lolkema JS, San Romão MV & Loureiro Dias MC (1996) Proton motive force generation by malolactic fermentation in *Leuconostoc oenos*. J. Bacteriol. in press

Sanders JW, Leenhouts KJ, Haandrikman AJ. Venema G & Kok J (1995) Stress response in *Lactococcus lactis*: cloning, expression analysis, and mutation of the lactococcal superoxide dismutase gene. J. Bacteriol. 177: 5254–5260

Schmitt P, Divies C & Merlot C (1990) Utilization of citrate by *Leuconostoc mesenteroides* subsp. *cremoris* in continous culture. Biotechnol. Lett. 12: 127–130

Schuldiner S, Shirvan A & Linial M (1995) Vesicular neurotransmitter transporters: from bacteria to humans. Physiol. Rev. 75: 369–392

Simon SM & Schindler M (1994) Cell biological mechanisms of multidrug resistance in tumors. Proc. Natl. Acad. Sci. USA 91: 3497–3504

Ten Brink B & Konings WN (1980) Generation of an electrochemical proton gradient by lactate efflux in membrane vesicles of *Escherichia coli*. Eur. J. Biochem. 111: 59–66

— (1982) Electrochemical proton gradient and lactate concentration gradient in *Streptococcus cremoris* cells grown in batch culture. J. Bacteriol. 152: 682–686

Ten Brink B, Otto R, Hansen UP & Konings WN (1985) Energy recycling by lactate efflux in growing and nongrowing cells of *Streptococcus cremoris*. J. Bacteriol. 162: 383–390

Van Veen HW, Bolhuis H, Putman M & Konings WN (1996a) Multidrug resistance in prokaryotes: molecular mechanisms of drug efflux. In: Kaback HR, Konings WN & Lolkema JS (Eds) Handbook of Biological Physics, Vol. II Transport Processes in Eukaryotic and Prokaryotic Membranes. Chapter VIII (pp. 165–185) Elsevier Science, Amsterdam

Van Veen HW, Venema K, Bolhuis H, Oussenko I, Kok J, Poolman B, Driessen AJM & Konings WN (1996b) Multidrug resistance mediated by a bacterial analog of the human drug transporter MDR1 Proc. Natl. Acad. Sci in press

Zaman GJR, Flens MJ, Van Leusden MR, De Haas M, Mülder HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ & Borst P (1994) Analysis of the expression of MRP, the gene for a new putative transmembrane. Proc. Natl. Acad. Sci. USA 91: 8822–8826